Dietary vitamin D supplementation attenuates immune responses of pigs challenged with rotavirus potentially through the retinoic acid-inducible gene I signalling pathway

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Abstract

In the present study, twenty-four Duroc ✕ Landrance ✕ Yorkshire (initial body weight (BW) of 21·82 (SEM 2·06) kg) cross-bred pigs were used to determine whether dietary vitamin D supplementation could confer protection against viral infections through the retinoic acid-inducible gene I (RIG-I) signalling pathway in pigs. Experimental treatments were arranged in a 2×2 factorial manner with the main effects of immune challenge (control *v.* porcine rotavirus (PRV) challenge) and dietary concentrations of vitamin D (200 and 5000 IU; where 1 IU of vitamin D is defined as the biological activity of 0.025 mg of cholecalciferol). The pigs were fed a diet containing 200 or 5000 IU vitamin D in the first week of the study period. On day 8, the pigs were orally dosed with 4 ml of Dulbecco's modified Eagle's medium/Ham's F-12 medium containing PRV or essential medium (control). Serum samples were collected on day 8 (pre-challenge), and 6 d after the PRV challenge, the pigs were killed to evaluate intestinal morphology and tissue gene expression following the last blood collection. Pigs challenged with PRV had decreased BW gain ($P < 0.01$), feed intake ($P < 0.01$), villus height ($P < 0.01$), faecal consistency ($P < 0.05$), and serum 1,25-dihydroxyvitamin D concentration ($P < 0.01$) and increased ($P < 0.01$) serum IL-2, IL-6 and interferon (IFN)-β concentrations. Vitamin D supplementation mitigated these effects. The mRNA expression of *RIG-I* ($P < 0.01$), IFN-β promoter stimulator 1 ($P < 0.01$), *IFN-β* ($P < 0.01$) and interferon-stimulated gene 15 (*ISG₁₅*) ($P < 0.01$) was up-regulated by the PRV challenge and vitamin D supplementation in the intestine. In conclusion, vitamin D supplementation could activate the RIG-I signalling pathway and thus alleviate the negative effects caused by PRV challenge.

Key words: Vitamin D: Rotavirus: Pigs: Retinoic acid-inducible gene I signalling

The innate immune system is the first line of defence against invading pathogens. Innate immune responses are initiated by pattern recognition receptors, which recognise specific structures expressed by micro-organisms[1–3]. Retinoic acid-inducible gene I (RIG-I), a type of pattern recognition receptor, plays a vital role in cytoplasmic viral double-stranded RNA recognition and innate immunity and in bridging innate and adaptive immune responses[4,5]. Upon recognition, RIG-I triggers signals through interferon-β promoter stimulator 1 (IPS-1, also termed MAVS/VISA/Cardif), which results in the activation of host transcription factors, including NF-κB and interferon regulatory factor 3, that orchestrate an early interferon (IFN)-independent transcriptional programme[6–8].

Innate immune responses to viral infections are critically dependent on the successful production of type I IFN[9].

Vitamin D belongs to the steroid hormone family. Vitamin D has two major forms, 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D3 (1,25(OH)₂D3). The major storage form of vitamin D is 25-hydroxyvitamin D, which is converted by the enzyme 1α-hydroxylase (CYP27B1, cytochrome P450, family 27, subfamily B, polypeptide 1) to the main active metabolite 1,25(OH)₂D3[10]. Vitamin D has conventionally been associated with bone mineralisation and Ca homeostasis. New evidence from epidemiological studies reveals that it also plays an important role in the regulation of the immune system, including immune responses to viral infections. Vitamin D

deficiency may predispose individuals to an increased risk of viral infections such as influenza[11], respiratory tract infections[12] and HIV[13]. A recent study has demonstrated that vitamin D has a direct anti-hepatitis C virus effect in an 'in vitro' infectious virus production system[14]. These results indicate that vitamin D mediates innate antiviral immune responses.

Rotavirus, a double-stranded RNA icosahedral RNA virus, is a major pathogen causing severe gastroenteritis in infants and young children, as well as in other young animals[15]. Recent studies have shown that the activation of the antiviral response by rotavirus depends on the RIG-I signalling pathway *in vitro* [16,17]. However, there are limited studies that have been carried out *in vivo*. As vitamin D represents a potentially useful intervention agent for combating viral infections, we assumed that the vitamin D-mediated innate antiviral immune response might be associated with the regulation of the RIG-I signalling pathway. Thus, we conducted the present study by employing a pig model of rotavirus infection to test this hypothesis.

Materials and methods

Animals and housing

The present experiment was carried out at the Experimental Farm of Sichuan Agricultural University. The animals were cared for according to the laws and regulations governing experiments with live animals in China. A total of twenty-four Duroc × Landrance × Yorkshire cross-bred pigs with an initial body weight of 21·82 (SEM 2·06) kg were randomly allocated to four treatment groups. The pigs were placed individually in metabolism cages (1·5 × 0·7 × 1·0 m) with a self-feeder and a nipple watering device, which were located in a temperature (25 ± 1°C)-controlled room. All the diets (Table 1) were formulated according to the National Research Council (NRC, 1998) requirements for all nutrients[18].

The pigs were fed the experimental diets for 7 d before the porcine rotavirus (PRV) challenge. Before the pigs were challenged with PRV, the experiment had a randomised complete-block design. After the pigs were challenged, the experiment was conceived as a 2 × 2 factorial arrangement based on the factors of challenge status (PRV challenged and unchallenged) and dietary vitamin D concentrations (200 and 5000 IU; where 1 IU of vitamin D is defined as the biological activity of 0.025 mg of cholecalciferol). In addition, the pigs challenged with PRV were fed separately from those orally dosed with essential medium to prevent mutual infection. Random distribution of two experimental diets was done between the pens of each room.

Rotavirus preparation

PRV (purchased from China Institute of Veterinary Drug Control) was a tissue culture-adapted Ohio State University (OSU) strain (ATCC #VR-893). The virus was propagated in IPEC-J2 cells as described previously[19]. The virus was pre-activated with 5 µg/ml trypsin (type IX, Sigma Chemical Company) for 30 min at 37°C. The IPEC-J2 cells were inoculated

Table 1. Composition of the diets used in the present study (as-fed basis)

Ingredients	g/kg
Maize	636·3
Soyabean meal	315·0
Soyabean oil	21·0
L-Lys·HCl	1·0
DL-Met	0·2
CaCO₃	7·2
CaHPO₄	11·3
Vitamin premix*†	0·5
Salt	2·5
Choline chloride	1·0
Mineral premix‡	4·0
Total	1000·0
Energy and nutrient levels§	
Digestible energy (MJ/kg)	14·23
Crude protein	185·0
Ca	7·0
Available P	3·2
Digestible Lys	10·6
Digestible Met	2·7

* The two experimental diets were supplemented with 200 IU of vitamin D_3 (control) or 5000 IU of vitamin D_3; where 1 IU of vitamin D is defined as the biological activity of 0.025 mg of cholecalciferol.

† Vitamin premix provided the following (per kg of diet): vitamin A, 5512 IU; vitamin D_3, 200 or 5000 IU; vitamin E, 24 mg; vitamin K_3, 3 mg; vitamin B_1, 3 mg; vitamin B_2, 6 mg; vitamin B_6, 3 mg; vitamin B_{12}, 24 µg; D-pantothenic acid, 15 mg; nicotinic acid, 30·3 mg; folic acid, 1·2 mg; biotin, 150 µg.

‡ Mineral premix provided the following (per kg of diet): Fe, 60 mg; Cu, 4 mg; Zn, 60 mg; Mn, 2 mg; Se, 0·10 mg; I, 0·14 mg.

§ Nutrient levels represent the calculated values.

with pre-activated rotavirus. After 60 min of incubation at 37°C, the inoculums were removed, and the monolayers were washed three times with PBS and then incubated at 37°C in Dulbecco's modified Eagle's medium/Ham's F-12 medium (DMEM/F12 medium). When an extensive cytopathic effect was observed by microscopy, the infected cultures were frozen and thawed three times and centrifuged (3000 *g* for 10 min), and supernatants containing the virus were stored at −80°C.

Virus titre determination

The tissue culture infective dose 50 ($TCID_{50}$) value was determined exactly as described previously[20]. Briefly, IPEC-J2 cells grown to 80–100 % confluency in ninety-six-well plates were infected with 50 µl aliquots of 1:10 serial dilutions (in DMEM/F12 medium) of virus samples (eight wells per dilution). After incubation for 4 d at 37°C in 5 % CO_2, cytopathic effects (cell destruction) were visualised by staining the remaining viable cells with crystal violet. Virus titres were calculated according to the method of Spearman[21] and are expressed as $\log_{10}(TCID_{50})$.

Rotavirus infection model

All animal procedures were approved by the Institutional Animal Care and Use Committee of Sichuan Agricultural University. The pigs were fed the experimental diets in the first week of the study period. To prevent intragastric viral inactivation, all the pigs were administered 5 ml of a 100 mM solution of sterile sodium bicarbonate before the PRV challenge[22].

On day 8 of the dietary treatments, the challenged pigs were orally dosed with 4 ml ($105 \cdot 8$ $TCID_{50}/100\,\mu l$) of rotavirus OSU strain and the unchallenged pigs with 4 ml of essential medium. The pigs were checked daily to evaluate their status after rotavirus challenge. Clinical signs (i.e. dehydration, apathy and diarrhoea) were monitored daily. Faecal consistency was recorded each morning at 08.00 hours by visual appraisal of each sample using a four-point scoring system and scored as follows: 0, solid; 1, semi-solid; 2, semi-liquid; 3, liquid. Infection was defined as the excretion of rotavirus in the faeces with or without the development of clinical symptoms, usually diarrhoea.

Blood samples were collected in the morning of days 8 and 14 and were centrifuged at 3000 g for 10 min. The isolated serum samples were stored at $-20°C$ until analysis. On day 14, all the pigs were killed with an intracardiac injection of sodium pentobarbital (150 mg/kg). The abdomen was immediately opened. The intestinal sections were identified according to the method of Wang et al.[23]. The mid-jejunum was isolated for histological analysis. The duodenum, jejunum and ileum were removed, flushed with ice-cold saline and then snap-frozen in liquid N_2 for further analysis.

Cytokine assays

The serum concentrations of IL-2, IL-6 and IFN-β were determined using commercially available swine ELISA kits (R&D Systems China Company Limited) according to the manufacturer's instructions. The detection limits of these ELISA were as follows: IL-2, 8 pg/ml; IL-6, 8 pg/ml; IFN-β, 20 pg/ml. The serum concentration of 1,25(OH)$_2$D3 was determined by ELISA (Immunodiagnostic Systems, Inc.). All the assays were run in duplicate using a plate reader.

Total RNA extraction and reverse transcription reaction

Total RNA was isolated using the TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The concentrations of total RNA were determined spectrophotometrically using a Beckman Coulter DU 800 (Beckman Coulter). The integrity of the RNA was verified by agarose gel electrophoresis. From each sample, 1 μg of total RNA was reverse-transcribed in a final volume of 20 μl using the PrimeScript® RT Reagent Kit with gDNA Eraser (TaKaRa) according to the manufacturer's protocol. RT products (complementary DNA) were stored at $-70°C$.

Real-time quantitative PCR

Real-time quantitative PCR were carried out using the CFX96 Real-Time PCR Detection System (Bio-Rad). The gene-specific primers used in the present study are listed in Table 2. The PCR mixture consisted of l μl of the first-strand complementary DNA sample, $0 \cdot 5$ μl of each of the forward and reverse primers from 10 μM stocks, 3 μl of diethylpyrocarbonate-treated water and 5 μl of 2 × SsoFast EvaGreen Supermix (Bio-Rad). Cycling conditions were as follows: 98°C for 10 s, followed by forty cycles of 98°C for 5 s, annealing at temperatures given in Table 2 for 10 s and 72°C for 15 s. A melting curve analysis was carried out after each real-time quantitative PCR to check and verify the specificity and purity of all PCR products. Each sample was amplified in triplicate. A standard curve was constructed using serial dilutions of one of the complementary DNA samples. The standard curve was drawn by plotting the natural log of the threshold cycle (C_t) against the natural log of the number of molecules. The C_t was defined as the cycle at which a statistically significant increase in the magnitude of the signal generated by the PCR was first detected. The standard curve of each gene was generated in duplicate and three times for obtaining reliable amplification efficiency values. The correlation coefficients (r^2) of all the standard curves were $>0 \cdot 99$ and the amplification efficiency values ranged between 90 and 110 %. The mRNA concentration of the target gene was normalised to that of the reference gene β-actin.

Table 2. Primers and annealing temperatures used in real-time quantitative PCR

Primer names	Sequence (5′–3′)	Product size (bp)	Annealing temperature (°C)	GenBank ID
RIG-I-QF	CCTCGGTGGCAGATGAAG	148	59·0	EU126659
RIG-I-QR	TCAGCGTTAGCAGTCAGAAG			
TLR3-QF	CTCGCTGATTCTCCTCTTCTC	150	59·0	NM_001097444
TLR3-QR	TCTCCATTCCTGTCCTGTGA			
IPS-1-QF	AGAAGCAGGACACAGAAC	95	59·0	JN644269
IPS-1-QR	GAAGGAGACAGTCGGAGA			
ISG$_{15}$-QF	ACCATTTCTGGCTGACTTTC	78	55·8	EU584557
ISG$_{15}$-QR	ACATAGGCTTGAGGTCATACT			
IFN-β-QF	AATCGCTCTCCTGATGTGTT	81	59·6	NM_001003923
IFN-β-QR	TTGCTGCTCCTTTGTTGGTA			
PRV-QF	TCAGTTCGTCAGGAATATGC	92	53·5	AF317123
PRV-QR	CTTGAAGGTGAGTAGTTGGT			
CYP27B1-QF	TGGTCAAGGAAGTGCTAAGA	86	55·8	DQ295065
CYP27B1-QR	AATGTAGTCACCCACACAAATG			
ACT-QF	TCTGGCACCACACCTTCT	97	59·0	U07786
ACT-QR	TGATCTGGGTCATCTTCTCAC			

RIG-I, retinoic acid-inducible gene I; QF, quantitative forward primer; QR, quantitative reverse primer; TLR3, Toll-like receptor 3; IPS-1, interferon-β promoter stimulator 1; ISG$_{15}$, interferon-stimulated gene 15; IFN, interferon; PRV, porcine rotavirus; CYP27B1, cytochrome P450, family 27, subfamily B, polypeptide 1; ACT, actin.

Statistical analysis

Data collected before the PRV challenge were analysed by one-way ANOVA using the general linear model procedure of the Statistical Analysis Package, SAS version 9.1 (SAS Institute, Inc.). Faecal consistency data were analysed as repeated measures using the MIXED procedure of SAS 9.1 according to the following model:

$$Y_{ijkl} = \mu + \alpha_i + \beta_j + (\alpha\beta_{ij}) + U_k + \omega_l + (\alpha\omega)_{il} + (\beta\omega)_{jl} + (\alpha\beta\omega)_{ijl} + \varepsilon_{ijkl},$$

where μ is the mean, α_i is the effect of immune challenge (i = PRV or essential medium), β_j is the effect of vitamin D (j = 200 or 5000 IU), $\alpha\beta_{ij}$ is the interaction between immune challenge and vitamin D, U_k is the pig (k = 1, 2,..., 24), ω_l is the time (days 1, 2,..., 6), $\alpha\omega_{il}$ is the interaction between immune challenge and time, $\beta\omega_{jl}$ is the interaction between vitamin D and time, $\alpha\beta\omega_{ijl}$ is the interaction between immune challenge, vitamin D and time, and ε_{ijkl} represents the random error. Growth performance, intestinal morphology, serum cytokine and gene expression data were analysed according to the model, but the effects of time and the interaction between time, immune challenge and vitamin D were omitted. When the effects of treatment were established ($P<0.05$), treatment least-squares means were separated using the probability of difference function adjusted by the Tukey–Kramer test. Random effect was used to account for the variation between the pigs. Results are expressed as least-squares means with their standard errors. Probability values <0.05 were used as the criterion for statistical significance.

Results

Growth performance

During the pre-challenge periods, there were no significant differences in the average daily gain, average daily feed intake (ADFI) and feed:gain ratio of the pigs. Compared with the unchallenged pigs, the 200 IU dietary vitamin D-supplemented pigs challenged with PRV had decreased average daily gain ($P<0.05$) and ADFI ($P<0.05$), whereas in the 5000 IU dietary vitamin D-supplemented pigs challenged with PRV, average daily gain and ADFI reduction was significantly alleviated (Table 3). The PRV challenge also led to a remarkable decrease in the feed:gain ratio ($P<0.01$). However, there were no significant effects of dietary supplementation with 5000 IU vitamin D on the feed:gain ratio. There was an interaction between dietary vitamin D levels and PRV challenge with regard to ADG and ADFI.

Faecal consistency

The effects of treatment on faecal consistency from day 8 to day 14 after the challenge are shown in Fig. 1. On day 6 after the challenge, the 200 IU dietary vitamin D-supplemented pigs challenged with PRV had reduced faecal consistency compared with the unchallenged pigs ($P<0.05$). Dietary supplementation with 5000 IU vitamin D improved the faecal consistency of the challenged pigs compared with dietary supplementation with 200 IU vitamin D, but not to the same extent as that observed in the unchallenged pigs ($P<0.05$).

Histomorphology

Compared with the unchallenged pigs, the 200 IU dietary vitamin D-supplemented pigs challenged with PRV had decreased villus height ($P<0.05$) and crypt depth ($P<0.05$) (Table 4). Dietary supplementation with 5000 IU vitamin D mitigated the challenge-induced damage to the intestinal epithelium ($P<0.05$). There was an interaction between dietary vitamin D levels and PRV challenge with regard to villus height ($P<0.01$). No difference was found in the crypt depth and villi:crypt ratio in the mid-jejunum among the treatment groups.

Serum IL-2, IL-6 and interferon-β concentrations

As shown in Fig. 2, the 200 IU dietary vitamin D-supplemented pigs challenged with PRV had significantly increased serum concentrations of IL-2 ($P<0.01$), IL-6 ($P<0.01$) and IFN-β ($P<0.01$) on day 14 compared with the unchallenged pigs.

Table 3. Effect of vitamin D (VD) supplementation and porcine rotavirus (PRV) challenge on the growth performance of pigs*

(Mean values with their standard errors, 1–7 d (*n* 12); 8–14 d (*n* 6))

Items	PRV (−) VD (200 IU)	PRV (−) VD (5000 IU)	PRV (+) VD (200 IU)	PRV (+) VD (5000 IU)	SEM	VD	PRV challenge	VD×PRV challenge
ADG (g)								
Days 0–7 (pre-challenge)	669·6	658·8	–	–	10·3	0·445	–	–
Days 8–14 (post-challenge)	669·4[c]	679·2[c]	430·6[a]	540·3[b]	14·0	<0·01	<0·01	<0·01
ADFI (g)								
Days 0–7 (pre-challenge)	1365·0	1364·5	–	–	20·0	0·303	–	–
Days 8–14 (post-challenge)	1350·1[c]	1398·5[c]	1067·5[a]	1251·5[b]	30·1	<0·01	<0·01	0·036
Feed:gain ratio								
Days 0–7 (pre-challenge)	2·04	2·07	–	–	0·03	0·179	–	–
Days 8–14 (post-challenge)	2·02[a]	2·06[a]	2·49[b]	2·32[b]	0·06	0·285	<0·01	0·080

ADG, average daily gain; ADFI, average daily feed intake.
[a,b,c] Mean values within a row with unlike superscript letters were significantly different ($P<0.05$).
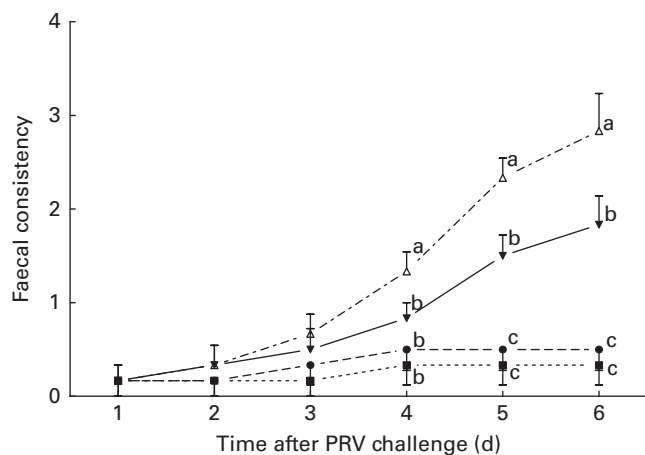* 1 IU of vitamin D is defined as the biological activity of 0.025 mg of cholecalciferol.

Fig. 1. Faecal consistency (experimental days 1–6) of pigs not challenged with PRV and fed a diet supplemented with 200 IU vitamin D (NONV, ●), pigs not challenged with PRV and fed a diet supplemented with 5000 IU vitamin D (NOHV, ■), pigs orally dosed with porcine rotavirus (PRV) and fed a diet supplemented with 200 IU vitamin D (CHNV, △), and pigs orally dosed with PRV and fed a diet supplemented with 5000 IU vitamin D (CHHV, ▼); where 1 IU of vitamin D is defined as the biological activity of 0.025 mg of cholecalciferol. Values are means, with their standard errors represented by vertical bars. [a,b,c] Mean values with unlike letters were significantly different within each day ($P < 0.05$).

Dietary supplementation with 5000 IU vitamin D decreased the concentrations of IL-2 ($P < 0.01$) and IL-6 ($P < 0.01$), but significantly increased that of IFN-β ($P < 0.01$) compared with dietary supplementation with 200 IU vitamin D.

Serum 1,25-dihydroxyvitamin D concentration and cytochrome P450, family 27, subfamily B, polypeptide 1 mRNA expression in the mid-jejunum

Data obtained for serum $1,25(OH)_2D3$ concentration and *CYP27B1* mRNA expression in the mid-jejunum are shown in Fig. 3. Compared with the 200 IU dietary vitamin D-supplemented pigs not challenged with PRV, the 200 IU dietary vitamin D-supplemented pigs challenged with PRV had a reduced serum concentration of $1,25(OH)_2D3$ ($P < 0.01$). Dietary supplementation with 5000 IU vitamin D increased the serum concentration of $1,25(OH)_2D3$ ($P < 0.01$). CYP27B1 is required for the conversion of 25-hydroxyvitamin D into $1,25(OH)_2D3$. The abundance of *CYP27B1* mRNA in the mid-jejunum was enhanced after the PRV challenge ($P < 0.01$). Dietary supplementation with

5000 IU vitamin D tended to increase the abundance of *CYP27B1* mRNA in the mid-jejunum. Furthermore, there was a significant effect of vitamin D level × PRV challenge interaction on the abundance of *CYP27B1* mRNA in the mid-jejunum.

Expression of retinoic acid-inducible gene I, Toll-like receptor 3, interferon-β promoter stimulator 1, interferon-β and interferon-stimulated gene 15 in the intestine

Data obtained for the mRNA expression of *RIG-I*, Toll-like receptor (*TLR*)3, *IPS-1*, *IFN-β* and *ISG₁₅* are summarised in Table 5. Compared with the 200 IU dietary vitamin D-supplemented pigs not challenged with PRV, the 200 IU dietary vitamin D-supplemented pigs challenged with PRV had an increased abundance of *RIG-I* mRNA in the duodenum ($P < 0.05$), jejunum ($P < 0.05$) and ileum ($P < 0.05$). Dietary supplementation with 5000 IU vitamin D significantly increased the abundance of *RIG-I* mRNA in the jejunum ($P < 0.05$) and ileum ($P < 0.05$) compared with dietary supplementation with 200 IU vitamin D. There was a significant effect of vitamin D level × PRV challenge interaction on the mRNA expression of *RIG-I* in the jejunum and ileum ($P < 0.01$). No effects of treatment were observed on the mRNA expression of *TLR3*.

Compared with the 200 IU dietary vitamin D-supplemented pigs not challenged with PRV, the 200 IU dietary vitamin D-supplemented pigs challenged with PRV had an increased abundance of *IPS-1* mRNA in the duodenum ($P < 0.05$), jejunum ($P < 0.05$) and ileum ($P < 0.05$). Dietary supplementation with 5000 IU vitamin D significantly increased the abundance of *RIG-I* mRNA in the duodenum ($P < 0.05$), jejunum ($P < 0.05$) and ileum ($P < 0.05$) compared with dietary supplementation with 200 IU vitamin D. There was a significant effect of vitamin D level × PRV challenge interaction on the mRNA expression of *IPS-1* in the jejunum ($P < 0.01$).

After the PRV challenge, the mRNA expression of *IFN-β* was increased in the duodenum ($P < 0.01$), jejunum ($P < 0.01$) and ileum ($P < 0.01$). Compared with the 200 IU dietary vitamin D-supplemented pigs challenged with PRV, the 5000 IU dietary vitamin D-supplemented pigs challenged with PRV had an increased abundance of *IPS-1* mRNA in the duodenum ($P < 0.05$), jejunum ($P < 0.05$) and ileum ($P < 0.05$). There was a significant effect of vitamin D

Table 4. Effect of vitamin D (VD) supplementation and porcine rotavirus (PRV) challenge on the villus height (VH; μm), crypt depth (CD; μm) and villi: crypt ratio (VCR) in the mid-jejunum of pigs*

(Mean values with their standard errors, n 6)

	PRV (−)		PRV (+)			Analysis by effect (P)		
Items	VD (200 IU)	VD (5000 IU)	VD (200 IU)	VD (5000 IU)	SEM	VD	PRV challenge	VD×PRV challenge
VH	482.8[b]	476.4[b]	391.8[a]	468.1[b]	13.56	<0.05	<0.01	<0.01
CD	209.7[b]	202.0[b]	176.0[a]	206.6[b]	8.54	0.18	0.09	0.06
VCR	2.33	2.47	2.30	2.29	0.13	0.58	0.45	0.33

[a,b] Mean values within a row with unlike superscript letters were significantly different ($P < 0.05$).
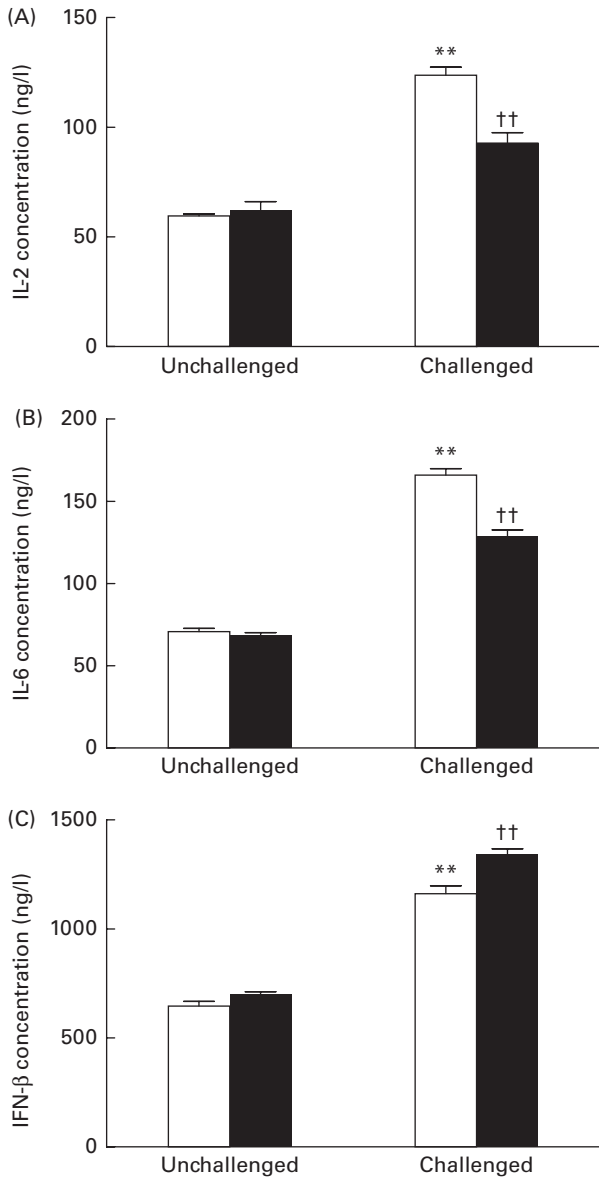* 1 IU of vitamin D is defined as the biological activity of 0.025 mg of cholecalciferol.

Fig. 2. Effect of vitamin D on the serum concentrations of (A) IL-2, (B) IL-6 and (C) interferon-β (IFN-β) of pigs challenged or not challenged with porcine rotavirus. □, 200 IU vitamin D; ■, 5000 IU vitamin D; where 1 IU of vitamin D is defined as the biological activity of 0.025 mg of cholecalciferol. Values are means, with their standard errors represented by vertical bars. ** Mean value was significantly different from that of the 200 IU vitamin D-supplemented group not challenged with PRV ($P < 0.01$). †† Mean value was significantly different from that of the 200 IU vitamin D-supplemented group challenged with PRV ($P < 0.01$).

level × PRV challenge interaction on the mRNA expression of *IFN-β* in the duodenum ($P < 0.01$) and jejunum ($P < 0.05$).

The abundance of *ISG15* mRNA in the duodenum, jejunum, and ileum ($P < 0.01$) was enhanced after the PRV challenge. Compared with the 200 IU dietary vitamin D-supplemented pigs challenged with PRV, the 5000 IU dietary vitamin D-supplemented pigs challenged with PRV had an increased abundance of *IPS-1* mRNA in the jejunum ($P < 0.05$) and ileum ($P < 0.05$). There was no effect of vitamin D level × PRV challenge interaction.

Discussion

Rotavirus is a major pathogen causing symptomatic gastroenteritis in infants and young children, as well as in other young animals[24,25]. The PRV used in the present experiment was a tissue culture-adapted OSU strain (ATCC #VR-893), which was passaged in IPEC-J2 cells. In the present study, we first used *in vivo* methods to elucidate the early interferon response to rotavirus infection and the effect of vitamin D on host–pathogen interactions.

The results of the present experiment were consistent with previous observations showing that PRV challenge caused a reduction in villus height, an increase in cytokine concentrations and finally a decrease in growth performance[26–28]. Rotavirus replicates in the enterocytes of the small intestine, inducing villous atrophy and malabsorptive diarrhoea[26]. Cytokines such as IL-6 are important markers of the progress of rotavirus infection in children[29]. In the present experiment, the serum concentration of IL-6 was significantly
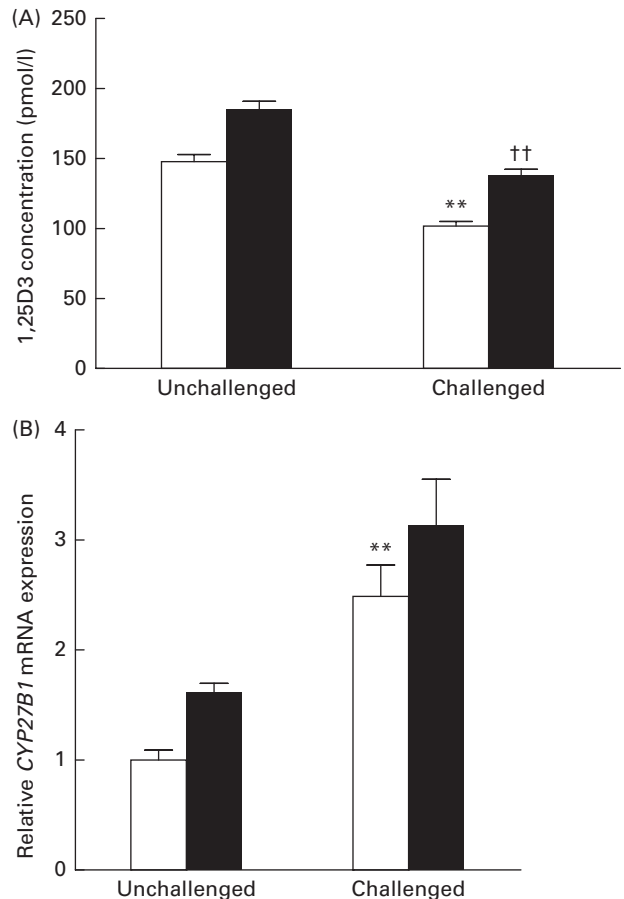

Fig. 3. Effect of vitamin D on the (A) serum 1,25-dihydroxyvitamin D (1,25(OH)₂D3) concentration and (B) cytochrome P450, family 27, subfamily B, polypeptide 1 (*CYP27B1*) mRNA expression of pigs challenged or not challenged with porcine rotavirus. □, 200 IU vitamin D; ■, 5000 IU vitamin D; where 1 IU of vitamin D is defined as the biological activity of 0.025 mg of cholecalciferol. Values are means, with their standard errors represented by vertical bars. ** Mean value was significantly different from that of the 200 IU vitamin D-supplemented group not challenged with PRV ($P < 0.01$). †† Mean value was significantly different from that of the 200 IU vitamin D-supplemented group challenged with PRV ($P < 0.01$).

Table 5. Effect of vitamin D (VD) supplementation and porcine rotavirus (PRV) challenge on the relative expression of retinoic acid-inducible gene I (*RIG-I*), Toll-like receptor (*TLR3*), interferon-β promoter stimulator 1 (*IPS-1*), interferon-β (*IFN-β*) and interferon-stimulated gene 15 (*ISG₁₅*) mRNA in the intestine of pigs*†

(Mean values with their standard errors, n 6)

	PRV(−)		PRV(+)			P		
Items	VD (200 IU)	VD (5000 IU)	VD (200 IU)	VD (5000 IU)	SEM	VD	PRV challenge	VD×PRV challenge
RIG-I								
Duodenum	1·00ᵃ	1·40ᵇ	1·55ᵇ,ᶜ	1·69ᶜ	0·08	<0·01	<0·01	0·09
Jejunum	1·00ᵃ	1·13ᵃ	2·50ᵇ	3·47ᶜ	0·09	<0·01	<0·01	<0·01
Ileum	1·00ᵃ	1·23ᵃ	3·16ᵇ	4·10ᶜ	0·09	<0·01	<0·01	<0·01
TLR3								
Duodenum	1·00	1·30	1·13	1·24	0·13	0·31	0·62	0·22
Jejunum	1·00	1·18	1·24	1·37	0·22	0·47	0·36	0·19
Ileum	1·00	0·91	1·08	1·03	0·10	0·51	0·35	0·83
IPS-1								
Duodenum	1·00ᵃ	1·20ᵃ	1·55ᵇ	1·99ᶜ	0·09	<0·01	<0·01	0·17
Jejunum	1·00ᵃ	1·16ᵃ	2·18ᵇ	3·41ᶜ	0·10	<0·01	<0·01	<0·01
Ileum	1·00ᵃ	1·02ᵃ	2·35ᵇ	2·84ᶜ	0·12	<0·05	<0·01	0·07
IFN-β								
Duodenum	1·00ᵃ	1·20ᵃ	1·92ᵇ	3·36ᶜ	0·13	<0·01	<0·01	<0·01
Jejunum	1·00ᵃ	1·07ᵃ	2·37ᵇ	3·24ᶜ	0·17	<0·01	<0·01	<0·05
Ileum	1·00ᵃ	1·06ᵃ	2·77ᵇ	3·31ᶜ	0·16	0·07	<0·01	0·14
ISG₁₅								
Duodenum	1·00ᵃ	1·05ᵃ	2·32ᵇ	2·58ᵇ	0·10	0·12	<0·01	0·30
Jejunum	1·00ᵃ	1·20ᵃ	2·77ᵇ	3·40ᶜ	0·14	<0·01	<0·01	0·13
Ileum	1·00ᵃ	1·22ᵃ	2·10ᵇ	2·64ᶜ	0·13	<0·01	<0·01	0·21

ᵃ,ᵇ,ᶜ Mean values within a row with unlike superscript letters were significantly different ($P < 0.05$).
* Tissues were collected in the morning of day 14.
† 1 IU of vitamin D is defined as the biological activity of 0.025 mg of cholecalciferol.

increased after the PRV challenge, while faecal consistency was significantly decreased. This result indicates that the PRV challenge model is useful for studying vitamin D-mediated innate antiviral immune responses.

Vitamin D is a steroid hormone that has long been known for its role in mineral metabolism and bone growth. The main active vitamin D ($1,25(OH)_2D3$) is thought to be a key component of non-classical vitamin D functions. In the present study, dietary vitamin D supplementation was found to increase serum $1,25(OH)_2D3$ concentration, while the PRV challenge was found to significantly decrease it, indicating that more amounts of $1,25(OH)_2D3$ are required to confer protection against viral infections in pigs. Apart from playing a role in bone mineralisation and Ca homeostasis, vitamin D also modulates the adaptive immune system by exerting direct effects on the activation of T cells and the phenotype and function of antigen-presenting cells, particularly of dendritic cells[30]. In addition, vitamin D also plays an important role in the regulation of the immune system, including immune responses to viral infections[11,12,13]. Due to the functions of vitamin D, it is easy to understand why pigs need more amounts of vitamin D under immune challenge conditions. The enzyme CYP27B1 is the key determinant in the synthesis of active vitamin D. This enzyme was first detected in the kidneys, where it has been found to be highly expressed. Extrarenal CYP27B1 has since been detected in numerous tissues, including the epithelial cells at various barrier sites[31] The presence of CYP27B1 in extrarenal tissues has been proposed as a mechanism by which vitamin D may exert a host of protective actions within these tissues[32]. In the present experiment, the mRNA expression of *CYP27B1* in the

mid-jejunum was also increased after the PRV challenge. This result is in agreement with that reported by Hansdottir et al.[32]. Hansdottir et al.[32] reported that polyinosinic-polycytidylic acid-treated primary airway epithelial cells have a significantly higher expression of *CYP27B1* mRNA than untreated cells. However, dietary supplementation with 5000 IU vitamin D in pigs challenged with PRV improved challenge-induced symptoms, as indicated by attenuated growth depression, decreased diarrhoea incidence, increased serum $1,25(OH)_2D3$ concentration and decreased intestinal damage. These results indicate that vitamin D has protective effects in pigs under PRV challenge conditions. In addition, the protective effects of vitamin D were also observed *in vitro*. Khoo et al.[33] reported that vitamin D down-regulates the proinflammatory cytokine response to *Mycobacterium tuberculosis* through pattern recognition receptors.

Mammalian innate immune responses to viral infections are critically dependent on a successful type I IFN response[9,34,35]. Type I IFN mediates antiviral activity by triggering the expression of IFN-stimulated genes, the products of which have diverse antiviral activities. RIG-I signalling is the main arm of innate immunity under double-stranded RNA virus challenge conditions[16,17,36]. In the present experiment, the mRNA expression of *RIG-I*, *IPS-1*, *IFN-β* and *ISG₁₅* in the intestine was up-regulated to various degrees after the PRV challenge. This is in close agreement with previous reports that RIG-I signalling through IPS-1 is required for the activation of IFN-β production by rotavirus-infected intestinal epithelial cells or mouse embryonic fibroblast cells and has a crucial role in the determination of the magnitude of rotavirus replication in the intestinal epithelium[16,17]. To our

knowledge, the present study is the first to demonstrate that the activation of the antiviral response by rotavirus is dependent on the RIG-I signalling pathway *in vivo*.

We also found that vitamin D is closely related to the RIG-I signalling pathway, which mediates innate immunity responses. The detection of pathogen-associated molecular patterns by pattern recognition receptors is critical for the initiation and coordination of immune responses against viral infections. To date, only the TLR have been reported to be associated with the immunological activities of vitamin D. A previous study has shown that the activation of TLR2/ TLR1 on human macrophages increases the synthesis of $1,25(OH)_2D3$ by these cells. This consequently induces an increase in the concentration of the antimicrobial peptide cathelicidin, leading to the destruction of intracellular *M. tuberculosis*[37]. Ovsyannikova *et al.*[38] reported the first evidence that polymorphisms in vitamin D receptor and *RIG-I* genes influence rubella vaccine-induced cellular immunity. In the present experiment, dietary supplementation with 5000 IU vitamin D increased the mRNA expression of *RIG-I*, *IPS-1*, *IFN-β* and *ISG_{15}*. Thus, the present results also suggest that the immunomodulatory effects of $1,25(OH)_2D3$ on rotavirus arise from its influence on the RIG-I signalling pathway. However, how vitamin D interacts with the RIG-I signalling pathway is not known still and requires further study.

In conclusion, dietary vitamin D supplementation attenuated weight gain and feed intake reduction caused by the PRV challenge. It is possible that the protective effects of vitamin D in pigs challenged with PRV are associated with the activation of the RIG-I signalling pathway through the up-regulation of *RIG-I*, *IPS-I*, *IFN-β* and *ISG_{15}* mRNA expression.

Acknowledgements

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